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No impact of neonicotinoids on male solitary bees *Osmia cornuta* under semi-field conditions

VERENA STROBL[1], SELINA BRUCKNER[2], SARAH RADFORD[3], SARAH WOLF[4], MATTHIAS ALBRECHT[4], LAURA VILLAMAR-BOUZA[1,5], JAKKRAWUT MAITIP[6,7], ELEONORA KOLARI[1], PANUWAN CHANTAWANNAKUL[6,8], GAÉTAN GLAUSER[9], GEOFFREY R. WILLIAMS[1,2,10], PETER NEUMANN[1,10] and LARS STRAUB[1,10]

[1]Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland, [2]Department of Entomology and Plant Pathology, Auburn University, Auburn, Alabama, U.S.A., [3]Faculty of Environment and Natural Resources, University of Freiburg, Freiburg, Germany, [4]Agroscope, Agroecology and Environment, Zurich, Switzerland, [5]European Food Safety Authority (EFSA), Pesticides Unit, Parma, Italy, [6]Bee Protection laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, [7]Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok, Rayong, Thailand, [8]Environmental Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, [9]Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Neuchâtel, Switzerland and [10]Agroscope, Swiss Bee Research Centre, Bern, Switzerland

Abstract. The ubiquitous use of agrochemicals is one driver for the ongoing loss of insect biomass and diversity. Data show that field-realistic concentrations of neonicotinoid insecticides can negatively affect both population density and the fitness of solitary bees. However, the underlying mechanisms for these effects remain poorly understood. Here, using an established semi-field experimental set-up and *Osmia cornuta* as a solitary bee model, we examined the effects of field-realistic concentrations of a common neonicotinoid insecticide (clothianidin) on male larvae and adults. Besides measuring lethal (i.e., overwintering success and adult survival) and sub-lethal endpoints (i.e., emergence mass and emergence duration), we examined, for the first time, potential effects on the male reproductive physiology of a solitary bee (i.e., sperm quantity and viability). The data revealed no significant effects on any of the measured response variables. This may be due to the low degree of clothianidin exposure (0.56 ng g^{-1}) and/or the apparent low susceptibility of solitary bee larvae to neonicotinoids. Furthermore, it is conceivable that ideal foraging conditions, combined with optimal weather and lack of other environmental stressors, may have improved the ability of bees to cope with the insecticide. To reliably assess and understand the environmental hazards of agrochemicals, a holistic approach, including laboratory, semi-field and field data is required. Knowledge of underlying mechanisms will help to mitigate the current global declines of insect populations.

Key words. Clothianidin, insecticides, lethal and sub-lethal effects, sperm traits.

Introduction

The ongoing global declines of insect abundance and biodiversity are alarming (Wagner, 2020). Agrochemicals, in particular neonicotinoid insecticides, acting singly and in combination

Correspondence: Verena Strobl, Institute of Bee Health, Vetsuisse Faculty, University of Bern, Schwarzenburgstrasse 161, 3003 Bern, Switzerland. Tel.: +41 (0) 631 57 65; Email: verena.strobl@vetsuisse.unibe.ch

with other factors, are considered to be a primary contributing factor (Woodcock *et al.*, 2017). The systemic broad-spectrum neonicotinoids are among the most widely applied insecticides (Hladik *et al.*, 2018), and both lethal and sub-lethal effects on various non-target species have been documented even at low exposure levels (Blacquière *et al.*, 2012).

Although the mode of action of neonicotinoid insecticides is well documented (Matsuda *et al.*, 2020), their impact on insect reproductive physiology remains poorly understood. This constitutes a major knowledge gap, as factors affecting reproductive physiology are likely to have a profound effect on an individual's fitness, and ultimately on the entire population (Lumley *et al.*, 2015). The few studies addressing the impact of neonicotinoid exposure on reproductive physiology have primarily focused on honey bees or female solitary bees (Williams *et al.*, 2015; Chaimanee *et al.*, 2016; Straub *et al.*, 2016; Wu-Smart & Spivak, 2018; Azpiazu *et al.*, 2019), whereas data for male wild bees are currently lacking. However, impaired male reproductive physiology may be a key mechanistic explanation for observed severe negative consequences of field-realistic neonicotinoid exposure on solitary bee fitness and population density (Sandrock *et al.*, 2014; Rundlöf *et al.*, 2015; Stuligross & Williams, 2020).

Here, we followed established protocols (Strobl *et al.*, 2019a, b) to examine the potential effects of neonicotinoids on male solitary bees, *Osmia cornuta*, under semi-field conditions. Based on previous studies (Straub *et al.*, 2016; Nicholls *et al.*, 2017; Sgolastra *et al.*, 2018), we hypothesized that neonicotinoid exposure would reveal no significant lethal effects on larvae or adults, but negative sub-lethal effects on adults (emergence mass and sperm traits).

Material and methods

The study was conducted at Agroscope-Reckenholz (Zürich, Switzerland), Neuchâtel Platform of Analytical Chemistry (Neuchâtel, Switzerland), and at the Institute of Bee Health (Bern, Switzerland), between September 2014 and May 2016. In early September 2014, oil-seed rape (OSR) was sown on an experimental field (128×57 m), which was divided into eight individual strips (each 3 m wide and 100 m long). According to a randomized split-plot design, OSR contained either neonicotinoids (hereafter, insecticide treatment) or not (hereafter, control). The exposed field contained OSR plants grown from seeds coated with clothianidin (seed coated; Modesto: clothianidin 400 g L^{-1}, Bayer CropScience AG, Germany), whereas, the control fields contained untreated OSR plants (Modesto®-free – Bayer Crop Science, U.K.).

In March 2015, a flight cage experiment was set up with 20 cages evenly distributed between the treatment groups ($n = 10$ per treatment) (Strobl *et al.*, 2019a). Each cage was equipped with a standardized nesting device for cavity-nesting *Osmia* bees (see Strobl *et al.*, 2019b). In April 2015, 200 female and 200 male *O. cornuta* cocoons [WAB – Mauerbienenzucht, Konstanz, Germany (http://mauerbienen.com/)] were placed in laboratory cages [10 females and 10 males each cage; BugDorm – Insect rearing cage ($47.5 \times 47.5 \times 47.5$ cm), Megaview Science Co. Ltd., Taiwan] within a greenhouse and maintained under natural day/night light conditions and constant room temperature (24 °C). Emerging individuals could freely mate for 4 days, and had *ad libitum* access to sucrose-solution provided via a universal cleaning sponge and pesticide-free honey bee collected corbicular pollen via a petri dish to ensure ovary maturation (Wasielewski *et al.*, 2011). Ten mated females and five additional males were randomly allocated to flight cages [($1.5 \times 1.5 \times 2.0$ m), Howitec Netting bv, Netherlands] with equal floral resources (52 OSR plants per m^2), which were blooming when the bees were added. To guarantee sufficient time for nest foundation, provisioning and egg-laying, bees were maintained for three weeks within their cages, mimicking the average life expectancy of females (Bosch & Vicens, 2006). Then, all nests were sealed and remained within the cages for an additional month to prevent interference with larval development. Thereafter, all nests were wrapped in polyester mesh net bags to avoid parasitism and protected from rain and direct sunlight. In October 2015, all tubes were opened and cocoons were stored individually in glass tubes for five months at 2 °C and complete darkness to undergo diapause (Krunic & Stanisavljevic, 2006).

In March 2016, offspring sex was determined according to cocoon size; 90 male cocoons (45 per treatment group) were randomly selected and exposed to emerging conditions following Strobl *et al.*, 2019b. Please refer to Strobl *et al.* (2019a) for the fate of the remaining cocoons. For the first 24 h, emergence times were recorded hourly and thereafter every 24 h. Cocoons with bees that did not emerge within two weeks were opened to assess if the bee inside was dead or alive. Only non-symptomatic bees [e.g., free of parasite infestations or possible clinical symptoms of a disease (Seidelmann, 2006)] were weighed to the nearest 0.1 mg (Mettler Toledo AT400), individually placed in a cage at room temperature provided with sucrose solution and pesticide-free honey bee collected corbicular pollen ad libitum (Strobl *et al.*, 2019b). Mortality rates were recorded daily. The assay was terminated after four days (96 h), when males are sexually mature and typically mate (Krunic & Stanisavljevic, 2006; Strobl *et al.*, 2019b). Then, 32 randomly chosen surviving individuals from both treatment groups were assessed for sperm quantity and viability (Straub *et al.*, 2016; Strobl *et al.*, 2019b). Lastly, to determine clothianidin residues in the pollen-nectar provisions, we performed ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) (Mitchell *et al.*, 2017). The residue values were determined by calculating the average of all tested samples per treatment ($N_{control} = 11$; $N_{insecticide} = 13$).

All data were tested for normality (Shapiro–Wilk's test), whereas homogeneity of variances was confirmed by visually inspecting residual plots. Generalized linear mixed models (GLMMs) with random intercepts were fitted using STATA16®, wherein individuals were considered independent units and treatment (insecticide vs. control) as the explanatory (fixed) term. Random effects were incorporated whenever applicable (see Supporting information, Table S1). A stepwise backward elimination approach was applied for each multiple regression model, where the model of best fit was chosen based upon the Akaike information criterion (AIC) and the Bayesian

information criterion (BIC). A logistic GLMM was applied to test for treatment differences for the binary outcome variable emergence success using the function *melogit*, whereby the conditional distribution of the regression was assumed to be Bernoulli. The remaining outcome variables, besides sperm viability and cumulative survival, were modelled with LMMs of the Gaussian family. As sperm viability is a score ranging from 0% to 100%, an ordered logistic model was fit using the function *meologit*. Survival data were fit using the *mestreg* function for multilevel survival models. A summary of statistical models, including the fixed and random effects are provided in Supporting information, Table S1.

Results

A summary of results for all outcome variables is given in Table 1. Clothianidin-treated fields had an average residue level of 0.56 ng g^{-1}, whereas the control fields showed residue levels of clothianidin below the limit of quantification (<0.08 ng g^{-1}). For both treatments, no signs of physical abnormalities or parasitism were observed. The data revealed no significant treatment effect for either lethal (i.e., overwintering success and adult survival) or sub-lethal endpoints (i.e., emergence time, emergence mass, sperm traits) (all *P*-values >0.16; see Supporting information, Table S1).

Discussion

Our data revealed no significant differences for any measured lethal or sub-lethal endpoint when clothianidin-exposed male *Osmia cornuta* were compared to controls. This indicates that the potential impact of neonicotinoids may not be detectable under the semi-field conditions of our experiments.

The detected residue values are in line with previous field findings (Rolke *et al.*, 2016). Further, the observed overall high overwintering and emergence success suggest optimal development despite mono-floral nutrition and neonicotinoid presence. Likewise, our data are in line with studies on closely related species (i.e. *Megachile rotundata*, *Osmia bicornis*, and *Osmia lignaria*) that neonicotinoid exposure at concentrations up to 30 ng g^{-1} has no significant effect on larval development and overwintering success (Abbott *et al.*, 2008; Nicholls *et al.*, 2017). This may be due to the lower expression of neonicotinoid key target sites (i.e. nicotine acetylcholine receptors and mushroom bodies) in larvae compared to adults (Nicholls *et al.*, 2017). While honey bee, *Apis mellifera*, data suggest larval exposure to neonicotinoids can cause physiological and morphological changes in adults (Friedli *et al.*, 2020), our data provided no evidence of such changes under the given conditions for *O. cornuta*. This may be due to different life histories of the studied bees (workers in *A. mellifera* vs. sexuals in *O. cornuta*), varying chemical exposure (combined thiamethoxam and clothianidin vs. clothianidin-only), species-specific ability to cope with toxins, or different treatment application and exposure to clothianidin (2.1 ng g^{-1} vs. 0.56 ng g^{-1}). While effects of neonicotinoid exposure on both fitness and population density of solitary bees have been shown (Sandrock *et al.*, 2014; Rundlöf *et al.*, 2015), differences due to experimental conditions (laboratory, semi-field, field), choice of crop (spring- vs. winter-sown OSR), local weather or genetics between tested species/populations may have masked effects in this study. Therefore, standardized methods are urgently required to promote reproducibility and direct comparisons of findings among different laboratories (Carreck *et al.*, 2020). Ultimately, this will then facilitate the identification of potential underlying mechanisms responsible for insect declines. In addition, such standardized methods and protocols would undoubtedly improve future risk assessment schemes and allow risk managers to propose adequate actions and mitigation measures.

Simultaneous exposure to additional stressors (e.g., pathogens, food stress, fluctuating temperatures and humidity) during larval development may result in detectable negative effects of neonicotinoid insecticides on developing and adult solitary bees. Indeed, bees are likely to encounter a wide array

Table 1. Summary of results for all outcome variables of the effects of a neonicotinoid insecticide on male solitary bees, *Osmia cornuta*.

						95% confidence interval	
Variables	Treatments	Sample size (*n*)	Mean	S.E.	Median	Lower	Upper
Emergence success (%)	Control	45	93.33	3.7	100	90.4	100
	Neonicotinoid	45	86	5.1	100	86.4	100
Emergence time (h)	Control	42	8.17	1.17	5.5	2.44	8.55
	Neonicotinoid	39	8.57	1.05	8	5.4	10.59
Emergence mass (mg)	Control	42	58.04	1.94	56	51.17	60.83
	Neonicotinoid	39	61.79	2.39	63	57.11	68.89
Survival (h)	Control	45	90.43	2.64	96	89.13	96
	Neonicotinoid	45	86.6	3.51	96	86.53	96
Sperm quantity (thousands)	Control	32	152.31	15.67	156	117.48	194.52
	Neonicotinoid	32	189.54	18.45	184	138.6	229.39
Sperm viability (%)	Control	32	61.16	3.5	66.17	57.31	75.02
	Neonicotinoid	32	61.78	2.49	62.96	56.81	69.11
Total living sperm (thousands)	Control	32	96.15	11.59	84	55.82	113.64
	Neonicotinoid	32	118.52	13.15	105	72.24	137.95

of stressors simultaneously in their natural habitats, and even low concentrations of neonicotinoids in combination with other factors are known to cause detrimental effects (Sánchez-Bayo & Wyckhuys, 2019). Such stressor combinations were unlikely in our experiment as the bees in the flight cages and later under laboratory conditions were maintained under favourable conditions (e.g., free of parasites and predation, optimal weather, single pesticide exposure, *ad libitum* feeding, minimal foraging distance, etc.). Ultimately, the impact of neonicotinoids is likely to vary depending on genetics and on the given environmental conditions and may be difficult to detect if those conditions are favourable. To reliably assess the potential environmental hazards of any given chemical, and prevent false-negative results, a holistic approach combining laboratory, semi-field and field data is required. Therefore, to better understand potential mechanisms driving global insect declines, especially concerning the role of agrochemicals, it appears urgent to address possible effects of these xenobiotics on male insects.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Summary of statistical methods and results of the neonicotinoid insecticide on male solitary bees, *Osmia cornuta*. Summary of all the models applied and the derived *z*-ratios and *P*-values for all fixed and random factors for each assessed outcome variable.

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Author contributions

M.A., P.N., G.R.W., and L.S. designed the experiment; V.S., S.W., S.R., L.S., N.K., L.V.-B., S.B., and J.M. collected field and laboratory data; M.A., P.C., and P.N. provided materials and reagents; L.S. and V.S. designed the statistical analysis; V.S., G.R.W., P.N., and L.S. analysed the data and wrote the manuscript. All authors edited and approved the manuscript.

Data availability statement

The complete data can be found on Dryad® repository (https://doi.org/10.5061/dryad.573n5tb63).

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